

**AMENDMENTS TO THE SPECIFICATION**

Please replace the paragraph beginning on page 1, line 3, with the following rewritten paragraph:

The research in this patent application was supported in part by National Institutes of Health grants CA ~~26380~~ 47956 and CA 60381. The United States government has certain rights in the invention.

Please replace the paragraph beginning on page 3, line 17, with the following rewritten paragraph:

In accordance with preferred embodiments, the anticode oligomer is substantially complementary to a strategic site in the pre-mRNA sense strand or substantially complementary to the mRNA. A preferred strategic site is the translation-initiation site of the pre-mRNA coding strand. Alternative strategic sites include coding sites for splicing, transport or degradation. The subject anticode oligomer either in its "native," unmodified form--oligonucleotide--or as a derivative, is brought into contact with the target lymphoma or leukemia cells. For *in vivo* therapeutic use, a derivative of the "native" oligonucleotide, such as the phosphorothioate form is preferable since it is believed that these forms are more resistant to degradation, notwithstanding the fact that response times to some analogues, such as the phosphorothioate analogs, has been found to be somewhat slower than to the "native" form of the oligonucleotide.

Please replace the paragraph beginning on page 6, line 22, with the following rewritten paragraph:

Figure 2 shows graphs of the concentration dependence of inhibition of cell proliferation by antisense normal and phosphorothioate oligodeoxynucleotides. Oligodeoxynucleotide additions to cultures included TI-AS phosphorothioate (○ and ●; two separate experiments), TI-S phosphorothioate (▲), TI-AS normal (□), and TI-S normal (Δ).

Please replace the paragraph beginning on page 7, line 17, with the following rewritten paragraph:

Figures 8 (a) - (c) show bcl-2 antisense oligodeoxynucleotides producing sequence-specific reductions in bcl-2 mRNA and bcl-2 protein and producing increased sensitivity of SU-DHL-4 cells to cancer chemotherapeutic drugs.

Please replace the paragraph beginning on page 7, line 25, with the following rewritten paragraph:

Figures 10 (a) and (b) (a-b) shows reduction of chemoresistance of RS11846 cells from inducible bcl-2 antisense expression from an expression plasmid.

Please replace the paragraph beginning on page 8, line 21, with the following rewritten paragraph:

The anticodon oligonucleotides and analogs thereof may be RNA or DNA, or analogs of RNA or DNA, commonly referred to as antisense oligomers or antisense oligonucleotides. Such RNA or DNA analogs comprise but are not limited to 2'-OO'-alkyl sugar modifications, methylphosphonate, phosphorothiate, phosphorodithioate, formacetal, 3'-thioformacetal, sulfone, sulfamate, and nitroxide backbone modifications, and analogs wherein the base moieties have been modified. In addition, analogs of oligomers may be polymers in which the sugar moiety has been modified or replaced by another suitable moiety, resulting in polymers which include, but are not limited to, morpholino analogs and peptide nucleic acid (PNA) analogs (Egholm, et al. Peptide Nucleic Acids (PNA)--Oligonucleotide Analogues with an Achiral Peptide Backbone, (1992)).

Please replace the paragraph beginning on page 9, line 4, with the following rewritten paragraph:

Anticodon analogs may also be mixtures of any of the oligonucleotide analog types together or in combination with native DNA or RNA. At the same time, the oligonucleotides and









Please replace the paragraph beginning on page 18, line 10, with the following rewritten paragraph:

B. Measurement of Cellular Growth. Growth of cell lines cultured in the presence or absence of anticodon oligomers was measured by two methods: cell counts using a hemocytometer; and DNA synthesis by assaying [ $^3\text{H}$ ]-thymidine incorporation essentially as described in Reed et al., *J. Immunol.*, 134:314-319 (1985). Briefly, cells were cultured in 96-well flat-bottomed microtiter plates (Falcon) at 0.2 ml/well. At appropriate times, cells were resuspended, 25  $\mu\text{l}$  removed from cultures for cell counting, and this volume replaced with 25  $\mu\text{l}$  of 20 UCi/ $\mu\text{L}$  [ $^3\text{H}$ ]-thymidine (specific activity 6.7 Ci/mmol) (New England Nuclear). Microtiter cultures were then returned to 37° C. and 95% air: 5%  $\text{CO}_2$  atmosphere for 8 hours before lysing cells on glass filters and determining relative levels of [ $^3\text{H}$ ]-thymidine incorporation into DNA by scintillation counting. Cell counts were performed in the presence of trypan blue dye to determine the concentration of viable cells in duplicate microcultures.

Please replace the paragraph beginning on page 18, line 29, with the following rewritten paragraph:

MTT [3-(4,-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] dye reduction assays were performed by the method of Tada, et al. J. Immunol. Methods 93, 157, (1986), and confirmed to be within the linear range of the assay under the conditions described here. The number of viable cells per well was extrapolated from standard curves that were included with each assay and that consisted of serial two-fold dilutions of exponentially growing SU-DHL-4 cells in HL-1 medium, beginning with  $10^6$  cells/ml (0.2 mlmi/well). Samples were assayed in triplicate and the OD600<sub>nm</sub> for a media/reagent blank was subtracted from all values prior to calculations.

Please replace the paragraph beginning on page 20, line 10, with the following rewritten paragraph:





To avoid experimental variation due to differences among lots of sera, 697 leukemic cells were cultured in serum-free conditions. When cultured at an initial seeding density of  $0.5 \times 10^6$  cells/ml, 697 cells achieved maximal DNA synthesis and cellular densities at 4-5 days. Addition of 25  $\mu$ M sense phosphorothioate oligodeoxynucleotide (TI-S) at the initiation of these cultures had little effect on 697 cell growth. In replicate cultures containing 25  $\mu$ M antisense phosphorothioate (TI-AS), however, some diminution in DNA synthesis was evident within 2 days and was maximal at 4-5 days. Maximal inhibition of 697 cell growth, as determined by cell counts, was seen at 6 days after initiation of cultures.

Please replace the paragraph beginning on page 27, line 18, with the following rewritten paragraph:

When 697 cells were initially seeded at  $0.2 \times 10^6$  cells/ml, the antisense phosphorothioate oligodeoxynucleotide, TI-AS, resulted in only slight inhibition at 2 days, attaining maximal suppression of DNA synthesis in these cultures at day 7. As with normal oligodeoxynucleotides, this inhibition by phosphorothioate oligodeoxynucleotides appeared to be mediated through non-cytotoxic mechanisms, since cellular viabilities did not decline until late in the course of culture. Compared with normal antisense oligodeoxynucleotides, therefore, phosphorothioate oligodeoxynucleotides had a slower onset of action.

Please replace the paragraph beginning on page 31, line 8, with the following rewritten paragraph:

For RNA blot analyses, polyadenylated mRNA was purified from normal NIH 3T3 cells and from cells stably transfected with expression constructs that produce either sense (3T3-alpha-S) or antisense (3T3-alpha-AS) recombinant bcl-2-alpha mRNAs, according to the method of 13. Approximately 5  $\mu$ g of mRNA was subjected to RNA blot analysis, essentially as described in (16), using either  $^3_2P$ - $^{32}P$ -labeled hybridization probes derived from either human or murine bcl-2 sequences.





Please replace the paragraph beginning on page 37, line 14, with the following rewritten paragraph:

As shown in Figures 6 (a) and (b), PO and PS *bcl-2* antisense oligonucleotides produced specific concentration-dependent reductions in the levels of *bcl-2* proteins, without altering the levels of expression of HLA-DR (Figure 7) and other control antigens. At 150  $\mu$ M, for example, PO antisense oligodeoxynucleotide caused an approximately 75-95% reduction in *bcl-2* fluorescence, whereas the control sense oligodeoxynucleotide diminished *bcl-2* protein levels by only 10-20% (Figure 6(a)). Similarly, cultured 697 cells for 4 days with the PS antisense oligodeoxynucleotide at 25  $\mu$ M resulted in approximately 70% reduction in *bcl-2* fluorescence. In comparison, the sense PS oligodeoxynucleotide TI-AS inhibited *bcl-2* protein levels by only approximately 15%, as measured by this assay (Figure 6(b)).

Please replace the paragraph beginning on page 37, line 30, with the following rewritten paragraph:

In phosphorothioate oligodeoxynucleotides, one of the non-bridging oxygen atoms in each internucleotide phosphate linkage is replaced by a sulfur atom. This modification renders phosphorothioate oligodeoxynucleotides extremely resistant to cleavage by nucleases, Stein et al., *Nucl. Acids Res.*, 16:3209-3221 (1988). Despite the substitution of a sulfur atom for an oxygen, phosphorothioate oligodeoxynucleotides retain good solubility in aqueous solutions; hybridize well, though with some decrease in the melting temperature of RNA-oligodeoxynucleotides duplexes; and are synthesized conveniently by the widely employed method of automated oligodeoxynucleotides synthesis with phosphoroamidites.

Please replace the paragraph beginning on page 40, line 31, with the following rewritten paragraph:

MCF-7 is a human breast adenocarcinoma cell line that contains relatively high levels of *bcl-2* protein. The cells were cultured at 4,000 cells per well in 96-well microtiter plates in the

presence or absence of MP/PO oligomers. Relative cell numbers per well were then estimated by MTT assay, based on a standard curve prepared using freshly plated, untreated MCF-7 cells. The antisense (As) and scrambled (Sc) MP/PO oligomers were the same as those described in Example 165. Data represent the mean +/- standard deviation for determinations.

Please replace the paragraph beginning on page 41, line 9, with the following rewritten paragraph:

The results demonstrate sequence specific inhibition of growth of solid tumor cells by the claimed anticodon oligomer analogs.

Please replace the paragraph beginning on page 41, line 19, with the following rewritten paragraph:

DoHH2 lymphoma cells were treated with various concentrations of oligomers targeted to different sites on the bcl-2 mRNA. The ATG oligomer (SEQ ID NO:17) targets the translation initiation site, and is complementary to the first 6 codons of the open reading frame. The Dscore 23 and Dscore 72 oligomers (SEQ ID NOS:26 and 28, respectively) target sites in the 5' untranslated region of the mRNA. Sc oligomers (SEQ ID NOS:25, 27 and 29) represent negative controls having the same length and base composition but in scrambled order. All oligomers were prepared as phosphodiester (PO)/phosphorothioate (PS) chimeras, where only the last (3') two internucleoside linkages were phosphorothioates. Oligomers were added directly to cultures and relative numbers of viable cells were estimated by MTT assay 3 days later. Data in Figure 13 represent mean +/- standard deviation.

Please replace the paragraph beginning on page 43, line 1, with the following rewritten paragraph:

Antimetabolites include, but are not limited to, methotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, hydroxyurea, 20-chlorodeoxy adenosine.

Please replace the paragraph beginning on page 43, line 5, with the following rewritten paragraph:

Alkylating agents include, but are not limited to, cyclophosphamide, melphalan, busulfan, cisplatin, paraplatin, chlorambucil, and nitrogen mustards.

Please replace the paragraph beginning on page 43, line 8, with the following rewritten paragraph:

Plant alkaloids include, but are not limited to, vincristine, vinblastine, and VP-16.

Please replace the paragraph beginning on page 43, line 28, with the following rewritten paragraph:

The methods for preparing and purifying the antisense (AS) and scrambled (SC) 18<sup>2</sup>-mer oligonucleotides used for the present work are described above in General Methods and in Kitada et al. (Antisense R & D, 3:157 (1993)). Phosphodiester oligonucleotides were synthesized in a 10-15 micromole scale using phosphoroamidate chemistry with oxidation by iodine, and then purified using a C<sub>18</sub>-reverse phase column. In most cases, oligomers were additionally ethanol-precipitated five times to eliminate any nonspecific cytotoxic activity, and then dried and resuspended in sterile HL-1 medium (Ventrex Labs, Inc; Burlingame, Calif.) at 1-10 mM. The pH of this solution was adjusted using 1-10 M NaOH until the phenol red indicator dye in the media returned to its original color.

Please replace the paragraph beginning on page 46, line 11, with the following rewritten paragraph:

Aliquots of 1.5 ml of HL-1 serum-free medium (Ventrex Labs, Inc.) supplemented with 1 mM L-glutamine, 50 Units/ml penicillin, and 100 ~~μ~~g/ml streptomycin and either 5 ~~μ~~g of purified oligonucleotides or 30 ~~μ~~g of Lipofectin<sup>R</sup>. [1:1 w/w mixture of N-(1-(2,3-dioleoyloxy)propyl)-~~n,n,n~~N,N,N-trimethylammonium chloride (DOTMA) and

dioleoylphosphatidylethanolamine (DOPE)] were combined and added to  $0.75 \times 10^6$  SU-DHL-4 cells in 3 mls of HL-1 medium. Cells were then either cultured at  $37^\circ \text{C}$ . in a humidified atmosphere of 5%  $\text{CO}_2$  /95% air in 24 well plates (2 mls/well) for immunoblot and RT-PCR assays, or in 96-well flat-bottom microtiter plates (0.1 ml/well) for MTT assays. For cells in microtiter cultures, typically 0.1 ml of additional HL-1 media with or without various chemotherapeutic drugs was added after 1 day, and the cells were cultured for an additional 2 days before performing MTT assays.

Please replace the paragraph beginning on page 46, line 28, with the following rewritten paragraph:

Cells were washed once in PBS, lysed in a buffer containing 1% Triton X100, and samples normalized for protein content (25  $\mu\text{g}$ ) prior to size-fractionation of proteins by SDS-PAGE (12% gels) and transfer to nitrocellulose filters for immunoblot assays as described in Reed et al. Cancer Res. 51:6529 (1991). Preliminary experiments determined that aliquots of lysates containing 25  $\mu\text{g}$  of total protein produced results in the linear range of the assay. Blots were first incubated with 0.1% (v.v) of a rabbit antiserum directed against a synthetic peptide corresponding to amino-acids (aa) 41-54 of the human Bcl-2 protein, as shown in SEQ ID NO. 21 (id) followed by 2.8  $\mu\text{g}/\text{ml}$  biotinylated goat anti-rabbit IgG (Vector Labs, Inc.). Bands corresponding to p26-Bcl-2 were then visualized by color development using a Horseradish Peroxidase (HRP)-avidin-biotin complex reagent (Vector Labs, Inc) and 3,3'-diaminobenzidine (DAB). Stained blots were then incubated with a second anti-Bcl-2 antibody directed against aa 61-76 of the Bcl-2 protein (SEQ ID NO. 21) followed by 0.25  $\mu\text{Ci}/\text{ml}$   $^{125}\text{I}$ -protein A. Bcl-2 bands were excised from the blots and subjected to gamma-counting.

Please replace the paragraph beginning on page 47, line 25, with the following rewritten paragraph:

Anticodons specific reductions in the relative levels of bcl-2 mRNA were detected within 1 day by a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. See Figure 8A.

Please replace the paragraph beginning on page 48, line 3, with the following rewritten paragraph:

In Figure 8B, SU-DHL-4 cells were cultured with pairs of either PS (squares) or PO/PS (circles) As- and Sc-oligomers for 3 days. Relative levels of Bcl-2 protein were then measured using a quantitative immunoblot assay, as described above, and the data expressed as a percentage relative to cells treated with control Sc-oligomers. The inset shows immunoblot results for p26-Bcl-2 and a p75 $\beta$  cross-reactive (CR) band in a case where As-PO/PS oligomer produced a 41% relative decrease in Bcl-2 protein levels. In Figure 8C, 10<sup>-4</sup>M Ara-C, MTX, or DEX was added 1 day after addition of PS (squares) or PO/PS (circles) oligomers to cultures of SU-DHL-4 cells, and MTT assays were performed on day 3. Data are presented as a % control relative to cells cultured with drugs in the absence of any oligomers, and represent the results of 9 of 10 consecutive experiments [in one experiment, the MTT assay failed]. Similar results were obtained when dye exclusion assays were used to assess cell survival rather than MTT assay [not shown].

Please replace the paragraph beginning on page 50, line 5, with the following rewritten paragraph:

In the present work, preliminary studies demonstrated that more than 90% of SU-DHL-4 cells survived treatment for 4 days with high dose (10<sup>-4</sup>) Ara-C, MTX or DEX, presumably because of their high levels of bcl-2 protein (Not shown). At these concentrations, however, all drugs induced essentially complete inhibition of SU-DHL-4 cell proliferation, consistent with bcl-2 converting drugs from cytotoxic to cytostatic. Comparisons of AS and SC oligomers demonstrated that bcl-2 AS treatment markedly enhanced the sensitivity of these lymphoma cells to MTX and Ara-C, and to a lesser extent to DEX (Figure 8C).





Please replace the paragraph beginning on page 53, line 22, with the following rewritten paragraph:

To prepare the expression plasmid, a 0.91 kbp bcl-2 cDNA (ibid) was subcloned in either antisense (AS) or sense (S) orientation into a HindIII site downstream of a human metallothionein-IIA promoter in the plasmid pMEP-4 (Invitrogen, Inc.), which contains a hygromycin phosphotransferase gene and the EBNA-1 gene and origin of DNA replication from Epstein-Barr Virus for high copy episomal maintenance.

Please replace the paragraph beginning on page 53, line 30, with the following rewritten paragraph:

RS11846 cells ( $5 \times 10^6$ ) in Dulbecco's phosphate buffered saline containing 30 ug of plasmid DNA were electroporated (1500 uF, 270 V/cm) using a Cellject Electroporation System from EquiBio, Inc. Cells were returned to their usual culture media (RPMI-L 1640 supplemented with 10% fetal bovine serum, 1 mM L-glutamine, 50 Units/ml penicillin, and 100 ug/ml streptomycin) at  $2 \times 10^5$  cells per ml and cultured for 2 days before seeding cells at  $2 \times 10^5$  per ml in media containing 200  $\mu$ g/ml hygromycin. After 3 weeks of culture, the resulting bulk cell lines were passaged in successively higher concentrations of hygromycin in 200  $\mu$ g/ml increments until the concentration reached 1 mg/ml (about 4 weeks).

Please replace the paragraph beginning on page 54, line 10, with the following rewritten paragraph:

Hygromycin-resistant RS11846 cell were cultured in RPMI/10% serum media containing 0.5  $\mu$ M CdCl<sub>2</sub> and 3 days later immunoblot assays were performed using 25 ug protein/lane essentially as described in Tanaka S, et al. J. Biol. Chem. 268, 10920 (1993) and in Reed et al. Cancer Res. 51:6529 (1991)).

Please replace the paragraph beginning on page 55, line 4, with the following rewritten paragraph:

Preliminary experiments demonstrated that RS11846 cells tolerated the addition of up to 0.5 ~~micro~~ $\mu$ M CdCl<sub>2</sub> or to ~~micro~~ 50  $\mu$ M ZnCl<sub>2</sub> to cultures for one week, experiencing a slight decrease in growth rate but essentially no decline in percentage cell viability (Not shown).

Please replace the paragraph beginning on page 55, line 9, with the following rewritten paragraph:

In the absence of heavy metal induction, the relative levels of bcl-2 protein in RS11846 cells containing the control or bcl-2 AS plasmid were comparable, as determined by immunoblot assays (Not shown). When 0.5 ~~u~~ $\mu$ M CdCl<sub>2</sub> or 50 ~~u~~ $\mu$ M ZnCl<sub>2</sub> was added, reductions in bcl-2 protein became evident in the AS-expressing cells at 2 days and maximal inhibition of 30-40% was obtained at three to four days, relative to control RS11846 cells.

Please replace the paragraph beginning on page 55, line 18, with the following rewritten paragraph:

Figure 10A shows an example of immunoblot lot data derived from RS11846 cells ~~afater~~ after three days of exposure to 0.5 mM CdCl<sub>2</sub>, demonstrating reduced levels of bcl-2 protein in the AS-plasmid containing cells compared to RS11846 cells that harbored the control plasmid. The relative levels of a control mitochondrial protein F<sub>1</sub> -beta-ATPase were comparable in all cell lines, consistent with sequence-specific alterations in bcl-2 protein levels.

Please replace the paragraph beginning on page 55, line 27, with the following rewritten paragraph:

When RS11846 cells containing either the control or bcl-2-As plasmids were cultured or various times in 0.5 ~~u~~ $\mu$ M CdCl<sub>2</sub>, or 50 ~~u~~ $\mu$ M ZnCl<sub>2</sub>, no significant difference in the growth rates of these two cell lines was observed (Figure 8B). Thus, As-mediated reductions in Bcl-2 protein levels by themselves did not impair RS11846 cell proliferation or survival.

This listing of claims will replace all prior versions, and listings, of claims in the application:

**LISTING OF CLAIMS:**

Claims 1-52 (canceled)

Claim 53 (previously presented): An anticode oligomer complementary to bcl-2 mRNA consisting of from 18-35 bases and comprising the nucleotide sequence TCTCCCAGCGTGCGCCAT (SEQ ID NO:17).

Claims 54-69 (canceled)

Claim 70 (currently amended): An anticodon oligomer, wherein said anticodon oligomer is from 10 to 40 bases in length and is complementary to a portion of ~~SEQ ID NO:19~~ a human bcl-2 mRNA or a portion of a human bcl-2 primary transcript.

Claim 71. (canceled)

Claim 72 (currently amended): The anticode oligomer of Claim 70, wherein said anticode oligomer is an antisense oligonucleotide complementary to a splice acceptor site of a human bcl-2 primary transcript-~~SEQ ID NO:19~~.

Claim 73 (currently amended): The anticode oligomer of Claim 70~~1~~, wherein said anticode oligomer is an antisense oligonucleotide complementary to a splice donor site of a human bcl-2 primary transcript ~~SEQ ID NO:22~~.

Claim 74 (currently amended): The anticode oligomer of Claim 70, wherein said anticode oligomer is 10 to 40 bases in length and is complementary to a 5'-untranslated region of a human bcl-2 mRNA or a human bcl-2 primary transcript ~~SEQ ID NO:19~~.

Claim 75 (currently amended): A composition comprising the anticode oligomer of Claims 53, 70, ~~71~~, 72, 73 or 74; and a pharmaceutically acceptable carrier.

Claim 76 (previously presented): The anticodon oligomer of Claim 53, wherein said anticodon oligomer contains at least one phosphorothioate-modified nucleotide.

Claim 77 (previously presented): A composition comprising the anticodon oligomer of Claim 76; and a pharmaceutically acceptable carrier.

Claim 78 (previously presented): The anticodon oligomer of Claim 76, wherein said anticodon oligomer is a phosphodiester/phosphorothioate chimera.

Claim 79 (previously presented): The anticodon oligomer of Claim 76 wherein the oligonucleotide comprises at least 2 to 3 phosphorothioate linkages.

Claim 80 (previously presented): A composition comprising the anticodon oligomer of Claim 78 or 79; and a pharmaceutically acceptable carrier.

Claim 81 (previously presented): The anticodon oligomer of Claim 53, wherein said anticodon oligomer contains at least one phosphoramidate-modified nucleotide.

Claim 82 (currently amended): The anticodon oligomer of Claims 70, ~~71~~, 72, 73 or 74, wherein said anticodon oligomer contains at least one phosphorothioate-modified nucleotide.

Claim 83 (previously presented): A composition comprising the anticodon oligomer of Claim 82; and a pharmaceutically acceptable carrier.

Claim 84 (previously presented): The anticodon oligomer of Claim 82, wherein said anticodon oligomer is a phosphodiester/phosphorothioate chimera.

Claim 85 (previously presented): The anticodon oligomer of Claim 84 wherein the oligonucleotide comprises at least 2 to 3 phosphorothioate linkages.

Claim 86 (previously presented): A composition comprising the anticodon oligomer of Claim 84 and a pharmaceutically acceptable carrier.

Claim 87 (previously presented): A composition comprising the anticode oligomer of Claim 85 and a pharmaceutically acceptable carrier.

Claim 88 (currently amended): The anticode oligomer of Claim 70, ~~71~~, 72, 73 or 74, wherein said anticode oligomer contains at least one phosphoramidate-modified nucleotide.